AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

GENETIC DIVERSITY AND EVOLUTION

Carnivore Parvovirus Ecology in the Serengeti Ecosystem: Vaccine Strains Circulating and New Host Species Identified

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ABSTRACT Carnivore parvoviruses infect wild and domestic carnivores, and cross-species transmission is believed to occur. However, viral dynamics are not well understood, nor are the consequences for wild carnivore populations of the introduction of new strains into wild ecosystems. To clarify the ecology of these viruses in a multihost system such as the Serengeti ecosystem and identify potential threats for wildlife conservation, we analyzed, through real-time PCR, 152 samples belonging to 14 wild carnivore species and 62 samples from healthy domestic dogs. We detected parvovirus DNA in several wildlife tissues. Of the wild carnivore and domestic dog samples tested, 13% and 43%, respectively, were positive for carnivore parvovirus infection, but little evidence of transmission between the wild and domestic carnivores was detected. Instead, we describe two different epidemiological scenarios with separate routes of transmission: first, an endemic feline parvovirus (FPV) route of transmission maintained by wild carnivores inside the Serengeti National Park (SNP) and, second, a canine parvovirus (CPV) route of transmission among domestic dogs living around the periphery of the SNP. Twelve FPV sequences were characterized; new host-virus associations involving wild dogs, jackals, and hyenas were discovered; and our results suggest that mutations in the fragment of the *vp2* gene were not required for infection of different carnivore species. In domestic dogs, 6 sequences belonged to the CPV-2a strain, while 11 belonged to the CPV-2 vaccine-derived strain. This is the first description of a vaccine-derived parvovirus strain being transmitted naturally.

IMPORTANCE Carnivore parvoviruses are widespread among wild and domestic carnivores, which are vulnerable to severe disease under certain circumstances. This study furthers the understanding of carnivore parvovirus epidemiology, suggesting that feline parvoviruses are endemic in wild carnivores in the Serengeti National Park (SNP), with new host species identified, and that canine parvoviruses are present in the dog population living around the SNP. Little evidence of transmission of canine parvoviruses into wild carnivore species was found; however, the detection of vaccine-derived virus (described here for the first time to be circulating naturally in domestic dogs) highlights the importance of performing epidemiological research in the region.

KEYWORDS carnivore parvovirus, conservation, molecular epidemiology, multihost, population genetics, vaccine shedding, viral evolution, wildlife

The species *Carnivore protoparvovirus 1*, known colloquially as the carnivore parvoviruses (https://talk.ictvonline.org/) (1), is a member of the *Parvoviridae* family and includes the antigenic variants feline parvovirus (FPV) and canine parvovirus (CPV).

Citation Calatayud O, Esperón F, Cleaveland S, Biek R, Keyyu J, Eblate E, Neves E, Lembo T, Lankester F. 2019. Carnivore parvovirus ecology in the Serengeti ecosystem: vaccine strains circulating and new host species identified. J Virol 93:e02220-18. https://doi.org/10.1128/JVI.02220-18.

Editor Joanna L. Shisler, University of Illinois at Urbana-Champaign

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Received 12 December 2018
Accepted 18 March 2019
Accepted manuscript posted online 17 April 2019
Published 14 June 2019

Carnivore parvoviruses infect a wide variety of host species, with complex pathological and epidemiological outcomes. They have a broad tropism for mitotically active cells and, depending on the strain, the presence of coinfection with other pathogens, and specific characteristics of the host, such as age, species, and host immunity, can cause subclinical, acute, or, especially in young animals, lethal disease (2–4).

Carnivore parvoviruses have a global distribution and are present in apparently healthy individuals from almost all wild and domestic carnivore populations tested (5–7). In contrast, there are reports that implicate the introduction of these viruses into wild ecosystems with a decrease in naive populations (e.g., wolves [*Canis lupus*]) (8, 9). Despite this apparent ubiquity and variable pathogenicity, the understanding of carnivore parvovirus evolution, strain succession, and spread is based upon a limited number of studies, mostly involving diseased captive wild and domestic animals (2, 4). These complexities illustrate the difficulties of predicting the consequences of infection at an individual level and at a population level, especially in wild ecosystems, where multiple potential hosts may reside. In order to better understand which wild species are at risk of infection and optimize conservation measures, it is necessary to further investigate the dynamics of carnivore parvoviruses.

Although FPV and CPV share a recent common ancestor from the early 1900s (10) and are differentiated by only small genetic changes, they show several important differences. Known since the 1920s (11), FPV is primarily associated with infection in felines rather than canines (with the exception of foxes) and has also been shown to infect Herpestidae, Mustelidae, and Procyonidae (5, 12–14). In contrast, CPV, which was first reported in the 1970s (15), shows signs of a recent population expansion, and while infection is intimately linked with domestic dog (*Canis lupus familiaris*) populations (16), the virus has been described in a wide range of species, including felines (13, 14, 16–18).

CPV infections in dogs have resulted in the emergence of different antigenic variants or strains: the first strain, designated CPV-2 (15), appeared in 1978 and was unable to infect felines. It spread globally and within a few months killed many naive domestic dogs (19, 20). A further strain, named CPV-2a, appeared in 1980 and rapidly replaced CPV-2 worldwide. While differentiated from CPV-2 by only a few amino acid substitutions, the CPV-2a strain regained the ability to infect felines (21). The most recent strains, CPV-2b and -2c, emerged in 1984 and 2000, respectively, and have only one amino acid substitution each relative to CPV-2a (3). Today, FPV coexists in different parts of the world with CPV-2a, -2b, and -2c, with unknown consequences for wild carnivore populations.

In Africa, molecular studies of FPV and CPV in domestic animals were carried out in a limited range of countries (South Africa, Morocco, Tunisia, Ghana, and Nigeria) (22–25), and results are consistent with findings from other regions of the world: CPV-2a, -2b, and -2c strains have been circulating within domestic dogs, while the original strain, CPV-2, has not been detected. Furthermore, virus sequences generated from these studies showed a high level of similarity with strains circulating in the rest of the world, suggesting that a similar epidemiological scenario exists in Africa as elsewhere.

Even fewer studies have focused on the role that African wild animals play in carnivore parvovirus ecology, and these have been limited to serological analyses (26–29). These studies have played an important role in demonstrating that African wild carnivore species can be infected with carnivore parvoviruses. However, unlike genetic analyses, serological studies do not enable strain characterization, and because strong antigenic cross-reactions occur among carnivore parvoviruses, the presence of antibodies does not enable conclusions to be drawn about the strain of the infecting virus (30, 31). Therefore, despite some studies concluding that wild carnivores have been infected by CPV, it is perhaps more appropriate to conclude that seropositive wild carnivores have at some point been infected by an unspecified carnivore parvovirus strain.

Genetic techniques provide an opportunity to investigate carnivore parvovirus

TABLE 1 Percentages of samples from different wild carnivore families and species that were positive for parvovirus DNA, detected by real-time PCR

Family and species	Total no. of samples	No. of positive samples	% infected (95% CI)
Viverridae (combined)	8	6	75 (34.9–96.8)
African civet	5	4	80 (28.4–99.5)
Genet	3	2	66.7 (9.4–99.2)
Herpestidae	7	1	14.3 (0.4–57.9)
Mongoose	7	1	14.3 (0.4–57.9)
Felidae (combined)	52	6	11.5 (4.4–23.4)
Lion	44	6	13.64 (5.2–27.4)
Cheetah	6	0	0
Leopard	1	0	0
Serval	1	0	0
Canidae (combined)	51	5	9.8 (3.3–21.4)
Black-backed jackal	15	2	13.3 (1.7–40.5)
Wild dog	20	3	15 (3.2–37.9)
Bat-eared fox	15	0	0
Aardwolf	1	0	0
Hyenas (combined)	34	3	8.8 (1.9–23.7)
Spotted hyena	32	2	6.3 (0.8–20.8)
Striped hyena	2	1	50 (1.3–98.7)
Total	152	21	13.8 (8.7–20.3)

ecology with more precision (12). As with human parvovirus infection, carnivore parvovirus DNA is likely to persist after the clinical period (32, 33), increasing the potential for detecting viral infections in archived animal samples. Indeed, the polymorphic *vp2* gene, which encodes the protein responsible for binding the transferrin receptor (TfR) used in carnivore parvovirus host cell attachment, provides a good candidate for molecular analyses and has been used for strain discrimination and to trace viral origins (19, 34).

The Serengeti ecosystem is an important area for the study of carnivore parvovirus ecology. First, it hosts large and diverse wild carnivore populations, which can provide critical information about natural infection routes. Second, some species living in the system, such as African wild dogs, are endangered (35) and require protection. Third, as mass dog vaccination programs against rabies, canine distemper, and CPV have been performed around the periphery of the Serengeti National Park (SNP) since 1996, this environment provides an opportunity to investigate the implications of mass dog vaccination in wild and domestic carnivore populations.

The principal objective of this study was to investigate the molecular epidemiology of carnivore parvoviruses in domestic and wild carnivores of the Serengeti ecosystem. While serological studies have confirmed the presence of carnivore parvovirus infection in lions (36), hyenas (31), jackals (27), wild dogs, and domestic dogs (37), no molecular studies have yet been carried out to characterize circulating strains in a wide range of potential host species. Within this objective, we aimed to investigate the natural routes of infection in wild and domestic carnivores, the likelihood of cross-species transmission, and the potential transmission of carnivore parvovirus strains found in vaccinated domestic dog populations.

RESULTS

Results in wildlife. (i) Presence of infection in wildlife. The presence of carnivore parvovirus DNA was confirmed in 13.8% (confidence interval [CI], 8.7 to 20.3%) ($n = 21$) of samples and in 9 out of 14 wild carnivore species tested (Table 1). In four of the five species in which carnivore parvovirus DNA was not detected, the sample size was low (<7), precluding conclusive inference regarding absence. The species with the highest

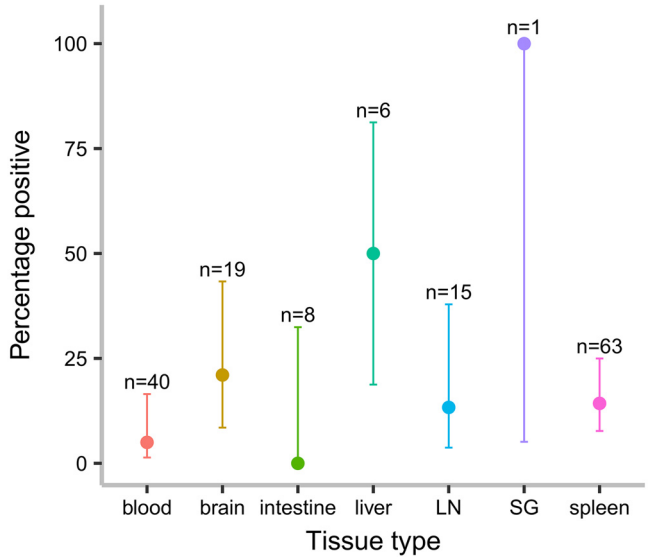

FIG 1 Percentages (and 95% confidence intervals) of wildlife samples that were parvovirus DNA positive for each tissue type (SG, salivary gland; LN, lymph node). The number of samples within each category is presented.

proportion of infected individuals was the African civet (*Civettictis civetta*), for which 80% (CI, 28.4 to 99.5%) ($n = 5$) of samples were positive. No infection was detected in the bat-eared fox (*Otocyon megalotis*), despite the relatively large sample size ($n = 15$). Of the seven different tissues analyzed, positive results were obtained in six (Fig. 1). The fecal sample from the positive lion, which was added *a posteriori*, was positive for infection.

The output from the binomial regression analysis investigating the determinants of carnivore parvovirus infection in the samples tested is given in Table 2. Liver samples (odds ratio [OR] = 17.8 [95% CI, 1.8, 218]; $P = 0.01$) and samples collected from Viverridae (OR = 17.6 [95% CI, 3.3, 118]; $P = 0.001$) were significantly more likely to be infected. The year of sample collection was not a predictor of infection (Fig. 2).

There was no association between the likelihood of sample infection and distance to the nearest building (OR = 1.0 [95% CI, 0.98, 1.04]; $P > 0.3$) or the SNP boundary (OR = 1.0 [95% CI, 0.99, 1.02]; $P > 0.1$) (Fig. 3).

(ii) Sequence analysis in wildlife. From a total of 21 positive wild carnivore samples, 13 *vp2* gene fragments were sequenced. Ten sequences consisted of 1,377

TABLE 2 Final regression output, investigating the determinants of the likelihood of detecting carnivore parvovirus DNA in the samples[a]

Determinant	Estimate	SE	*z* value	*P* (>|*z*|)
Intercept	−3.54	0.97	−3.65	0.0003***
Tissue				
Brain	1.99	1.03	1.92	0.05
Intestine	−16.48	2,039	−0.01	0.99
Liver	2.88	1.18	2.44	0.01*
Lymph node	1.48	1.11	1.33	0.18
Salivary gland	22.11	6,522	0.003	0.99
Spleen	0.59	0.92	0.64	0.52
Family				
Felidae	0.85	0.75	1.13	0.26
Hyaenidae	−0.05	0.85	−0.06	0.95
Viverridae	2.87	0.90	3.18	0.001**

[a]Significance is indicated (***, $P < 0.001$; **, $P = 0.001$; *, $P = 0.01$). The null deviance was 117.8 on 149 degrees of freedom. The residual deviance was 90.2 on 140 degrees of freedom. The AIC value was 110.2.

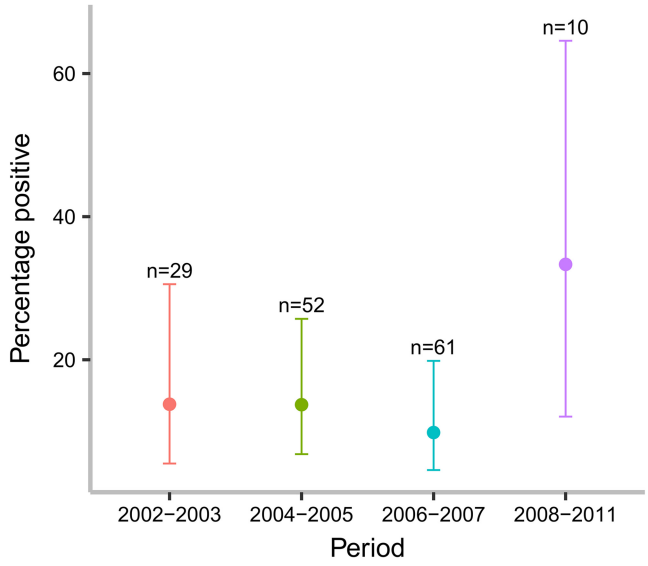

FIG 2 Percentages (and 95% confidence intervals) of wildlife samples that were parvovirus DNA positive for each time period. The number of samples within each category is presented.

nucleotides, and one sequence each consisted of 1,311, 1,088, and 699 nucleotides. Nucleotides previously used for the classification of carnivore parvoviruses (20, 38, 39) were present in all these isolates (detailed in Table 3): 12 isolates belonged to the FPV strain (three lions, two spotted hyenas [*Crocuta crocuta*], two African wild dogs [*Lycaon pictus*], two civets, one genet [*Genetta genetta*], one white-tailed mongoose [*Ichneumia albicauda*], and one black-backed jackal [*Canis mesomelas*]), while 1 isolate belonged to the CPV-2a strain (black-backed jackal). The FPV and CPV-2a isolates detected in black-backed jackals were found in two different individuals. This is the first time that FPV DNA has been detected in jackals, hyenas, African wild dogs, and white-tailed mongoose. Interestingly, the CPV-2a isolate was detected in the most recently obtained wild carnivore sample (2011). This isolate was the only sequence with intermediate features between FPV-like and CPV-like viruses (detailed below).

(iii) FPV in wildlife. Comparison of FPV sequences from this study showed nucleotide identities of 99.5 to 99.9% (mean, 99.75%; standard deviation [SD], 0.09%) and amino acid identities of 99.1 to 100% (mean, 99.61%; SD, 0.23%). This compares with a global BLAST search in GenBank in which no identical FPV sequences were found. Following comparison with the template strains from different years and locations, the nucleotide identity was 98.0 to 99.2% and the amino acid identity was 96.9 to 99.8%. The maximum amino acid variability of the study sequences was 0.9%, while the maximum amino acid variability between the study and the template sequences was 3.1%, suggesting that the study sequences were more closely related to each other than to sequences found elsewhere.

The FPV sequences detected in wild carnivore species in the Serengeti ecosystem shared two mutations at two different residue positions that distinguish them from FPV sequences described elsewhere. These mutations were located at amino acid position 303, where a Tyr replaced a Phe residue (F303Y), and at position 101, where a Thr replaced an Ile residue (I101T) (detailed in Table 3). Following comparison with the most similar FPV strains found in GenBank, the first mutation, F303Y, was found only in a cougar (*Puma concolor*) (United States, 1989; GenBank accession no. EU659113) and could have arisen independently in this individual. Residue 303 is located in the capsid surface area that contacts the host cell receptor, and as such, this position is subjected to evolutionary selective pressures (34). The second mutation, I101T, which emerged during the differentiation of CPV-2a from CPV-2 (40), has occasionally been reported in FPV sequences extracted from wild and domestic species from different years and

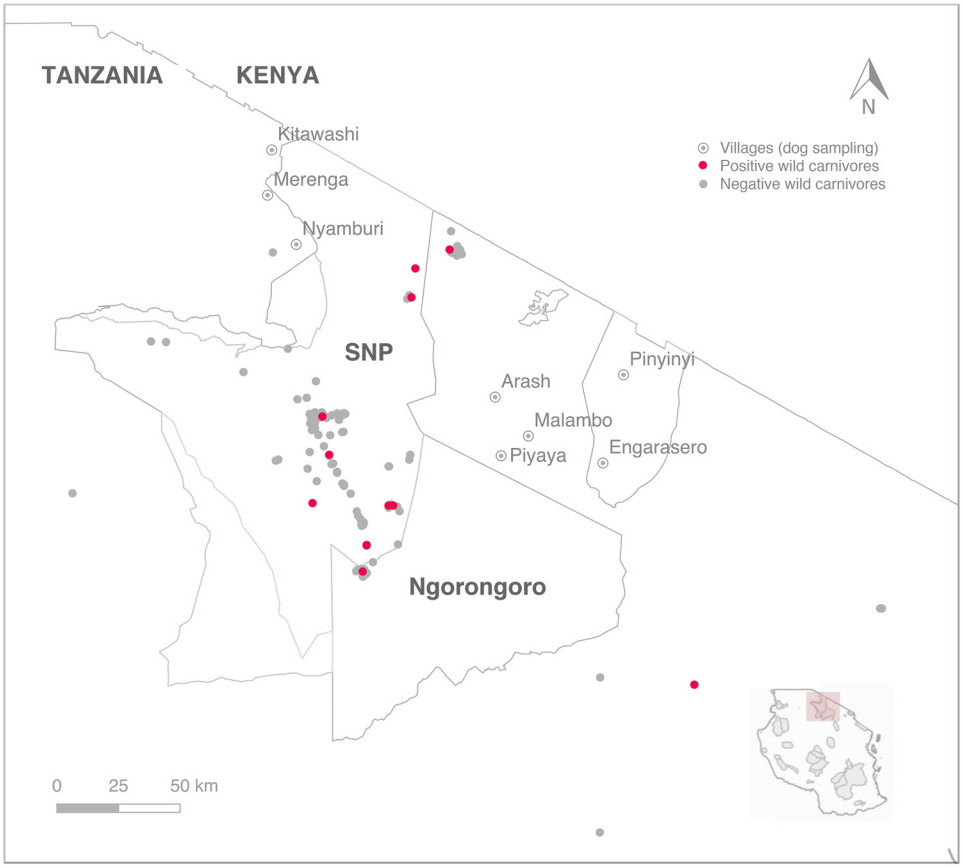

FIG 3 Map indicating the location of the Serengeti Maasai Mara ecosystem within Tanzania (inset). The shaded areas identify the Serengeti National Park (Serengeti), the Ngorongoro Conservation Area, and a number of unnamed game reserves. The locations of the villages where domestic dog samples were collected and the locations where positive and negative wildlife samples were collected are shown. (The map was created by O. Calatayud using a protected-areas layer from the IUCN and UNEP-WCMC [2019], The World Database on Protected Areas [WDPA] [www.protectedplanet.net].)

locations (e.g., GenBank accession no. MF069447, FJ440714, and KP682520). Polymorphic residue 101 lies just below the capsid surface and, together with residue 87, alters the antigenic structure and influences binding to feline and canine cells (41). Together, amino acid residues 303 and 101 determine host range, and the mutations detected in this study form a geographic cluster, as demonstrated by the phylogenetic network (see below).

A further mutation at position 20, where a Thr residue replaced an Ala residue (A20T), was present in three of eight FPV sequences containing this amino acid (belonging to two lions and a mongoose) (detailed in Table 3). This mutation was also found in four of the CPV sequences described in dogs sampled in this study (see below). Mutation A20T was not found in any of the most similar FPV strains found in GenBank, and information regarding this residue was lacking in the literature reviewed. We hypothesize that because residue 20 was located only a few residues away from the primer sequence, this mutation could be a sequencing error.

Five other single FPV sequence mutations were found (V83I, Q159H, H222P, V250M, and Q296H), each occurring in one sample only. Residues involved were not a strain type determinant, and no previous studies determining the effects of these substitutions were found.

Among the FPV sequences from this study, two pairs of amino acid sequences were pairwise identical. These sequences were obtained from the sample IDs (i) H414 (lion, liver, 2004) and H284 (white-tailed mongoose, spleen, 2008) and (ii) H450 (hyena, liver,

TABLE 3 Summary of the amino acid variation that characterizes the strains of carnivore parvoviruses and the important mutations that distinguish the sequences from this study[a]

				Amino acid residue																
Clade	Strain	Species	Year	20	80	87	93	101	103	219	297	300	303	305	321	323	347	375	386	426
FPV	Reference strains			A	K	M	K	I (+T)	V	I	S	A	F	D	N	D	A	D	Q	N
CPV-2				A	R	M	N	I	A	I	S	A	F	D	N	E (+N)	T	N	Q	N
CPV-2a				A	R	L	N	T	A	I	A (+S)	G	F	Y	K (+N)	E (+N)	A	D	Q	N
CPV-2b				A	R	L	N	T	A	I	A (+S)	G	F	Y	K (+N)	E (+N)	A	D	Q	D
CPV-2c				A	R	L	N	T	A	I	A (+S)	G	F	Y	N	N	A	D	Q	E
CPV-2	H494	Clf	2005	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H489		2005	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H504		2005	A	R	M	N	I	A	V	S	A	F	D	N	N	T	N	K	N
	H506		2005	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H469		2008	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H499		2008	T	R	M	N	I	A	V	S	-	F	D	N	N	T	N	K	N
	H488		2008	T	R	M	N	I	A	V	S	A	F	D	N	N	T	N	K	N
	H503		2008	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H476*		2008	-	-	-	-	-	-	-	S	A	F	D	N	N	T	N	K	N
	H327		2009	T	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
	H493		2009	A	R	M	N	T	A	V	S	A	F	D	N	N	T	N	K	N
CPV-2a	H491		2004	A	R	L	N	T	A	I	A	G	F	Y	N	N	A	D	Q	N
	H321		2005	T	R	L	N	T	A	I	A	G	F	Y	N	N	A	D	Q	N
	H501		2009	A	R	L	N	T	A	I	A	G	F	Y	N	N	A	D	Q	N
	H492*		2005	-	-	-	-	-	-	-	A	G	F	Y	N	N	A	D	Q	N
	H323*		2006	-	-	-	-	-	-	-	A	G	F	Y	N	N	A	D	Q	N
	H318*		2006	-	-	-	-	-	-	-	A	G	F	Y	N	N	A	D	Q	N
	H398*	Cm	2011	T	T	L	N	T	A	I	-	-	F	Y	N	D	A	D	Q	N
FPV	H382	Pl	2002	T	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H414	Pl	2004	T	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H227	Civ civ	2004	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H418	Cm	2005	-	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H450	Cc	2007	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H401	Cc	2007	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H439	Lp	2007	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H440	Pl	2008	-	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H284	Ia	2008	T	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H253	Civ.civ	2009	A	K	M	K	T	V	I	S	A	Y	D	N	D	A	D	Q	N
	H410*	Gg	2005	-	-	-	K	-	V	I	S	A	Y	D	N	D	A	D	Q	N
	H272*	Lp	2007	-	-	-	-	-	-	-	S	A	Y	D	N	D	A	D	Q	N
Vaccine CPV-2 (Intervet)	MG264079		2017	A	R	M	N	I	A	V	S	A	F	D	N	N	A	N	K	N
Vaccine CPV-2 (Pfizer)	EU914139		2006	A	R	M	N	I	A	K	S	A	F	D	N	N	A	D	R	N

[a]The 459-amino-acid fragment of the VP2 protein sequenced is represented. Blue indicates mutations among the domestic dog sequences, possibly introduced by the live-virus vaccine strain, and violet indicates important and repeated mutations found among the wildlife sequences * indicates an incomplete strain. Clf, *Canis lupus familiaris* (domestic dog); Cm, *Canis mesomelas* (black-backed jackal); Pl, *Panthera leo* (lion); Cc, *Crocuta crocuta* (spotted hyena); Civ. civ., *Civettictis civetta* (African civet); IA, *Ichneumia albicauda* (white-tailed mongoose); Lp, *Lycaon pictus* (wild dog); Gg, *Genetta genetta* (genet).

2007) and H253 (civet, spleen, 2009). Furthermore, five amino acid sequences (H414, H284, H450, H253, and H440) were differentiated by a nucleotide at only a single position (position 58), which encodes the amino acid residue at position 20, as discussed above.

(iv) CPV-2a from black-backed jackal H398. The sequence of H398 clustered phylogenetically with the CPV-2a sequences from dogs (see below). However, a single mutation at amino acid position 323 (an Asp residue replaced the CPV-2a-typical Asn or Glu residues [Table 3]) was present. As the amino acid at this position is exposed on the surface of the virus and controls the interaction with the canine transferrin receptor (TfR) (6), it is possible that this viral mutation would favor binding to a feline transferrin receptor.

Furthermore, we describe four additional amino acid mutations in this sequence: A20T, R80T, D99H, and D125Y (detailed in Table 3). Of these mutations, substitution A20T is shared by eight of our wildlife and domestic dog isolates.

(v) Phylogenetic analysis in wildlife. The phylogenetic tree (Fig. 4) indicated that the FPV strains detected in the wildlife species in this study have a common ancestor, formed a geographic cluster separated widely from other previously reported isolates, and are closely related, suggesting cross-species transmission.

Results in domestic dogs. (i) Presence of infection in dogs. The presence of carnivore parvovirus DNA was detected in 42.9% (CI, 30.5 to 56.0%) ($n = 26$) of the

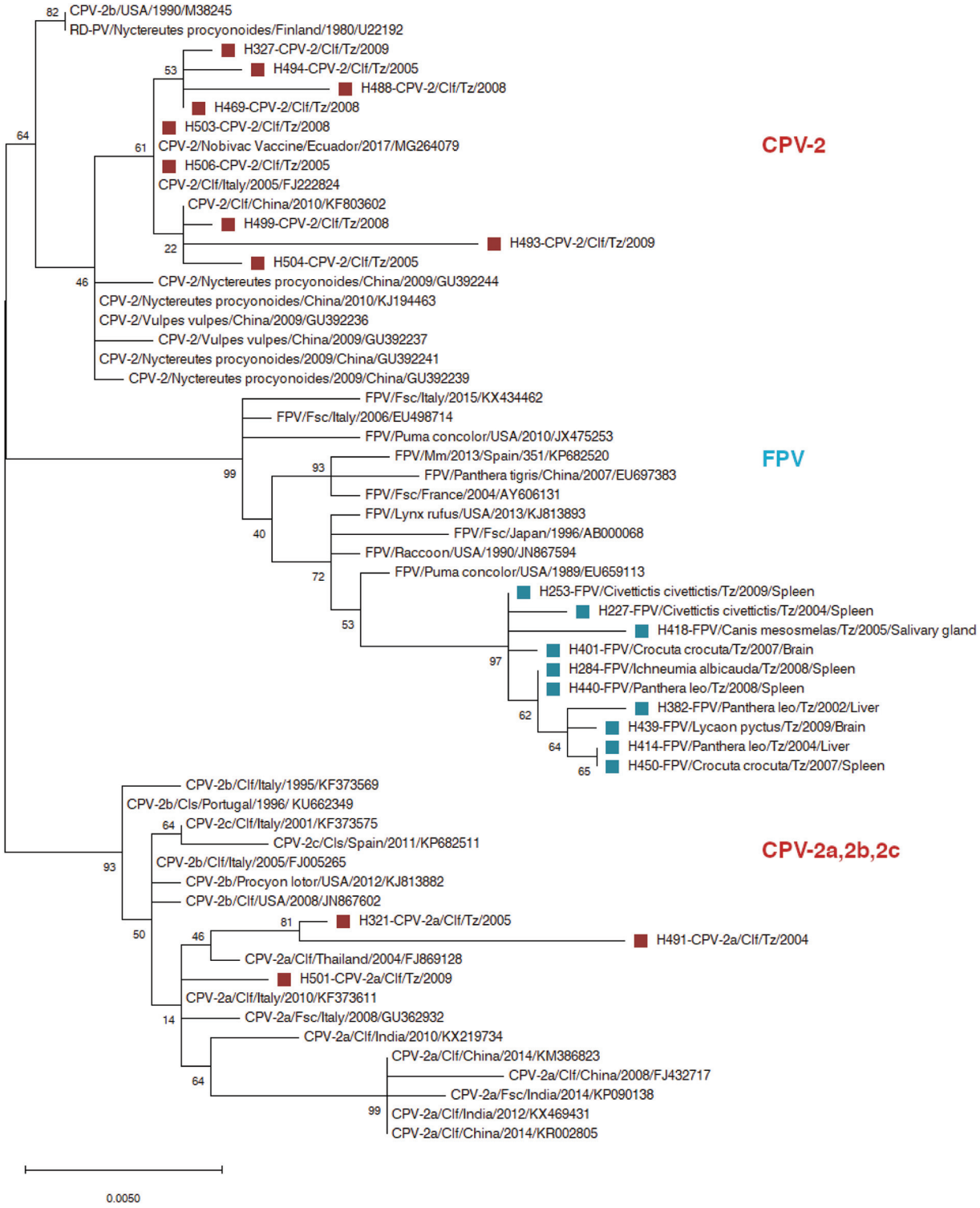

FIG 4 Phylogenetic tree constructed from the VP2 nucleotide sequences described in this study and in other parts of the world. Clf, *Canis lupus familiaris*; Fsc, *Felis silvestris catus*. All horizontal branches are drawn to a scale of nucleotide substitutions per site.

domestic dog samples assayed and in six of the eight villages (75%) in which sampling took place. The villages with the highest proportions of infected individuals were Merenga (2008) and Kitawasi (2005), in which 87.5% (CI, 47.3 to 99.7%) and 83.3% (CI, 35.9 to 99.6%) of dogs sampled were infected, respectively (Fig. 3 and 5). None of the

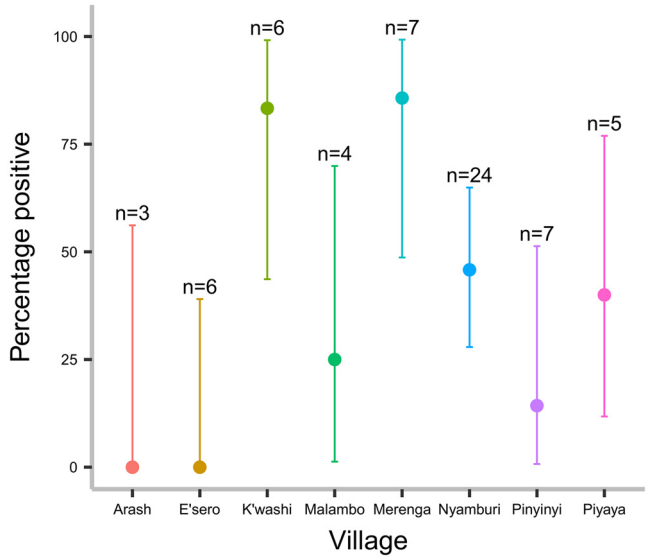

FIG 5 Percentages (and 95% confidence intervals) of domestic dog samples from each village that were positive for parvovirus DNA. The number of samples from each village is given.

factors studied (village, year of sampling, or age or gender of dog) were significant predictors of infection ($P > 0.4$).

(ii) Sequence analysis in dogs. From a total of 26 positive domestic dog samples, 13 isolates of 1,377 nucleotides and 4 isolates of approximately 700 nucleotides were obtained. Of these, 11 were classified as CPV-2, and 6 were classified as CPV-2a. Of the CPV-2 strains, three were found in samples from the village of Kitawasi (2005), six were from Merenga (2008), one was from Nyamburi (2009), and one was from Piyaya (2009). Of the CPV-2a strains, four were detected in Nyamburi (2005, 2006, and 2009), one was detected in Piyaya (2004), and one was detected in Kitawasi (2005) (Fig. 3 and 5).

CPV-2 and CPV-2a strains were differentiated using amino acid positions 87, 101, 219, 300, 305, and 375, which are considered determinant residue positions for the classification of the CPV strains (40, 42). Sixteen of the 17 sequences obtained from domestic dogs fulfilled this classification, with no intermediate viruslike features. An exception was the CPV-2 sequence obtained from isolate H493 (Table 3), which, apart from position 101, in which a Thr was replaced by Ile, presented all the residues that characterize the CPV-2 strain. Position 101 determines the antigenic structure and binding capabilities of the capsid (42), and a Thr residue at this position is typical of the CPV-2a, -2b, and -2c strains but has also been described in FPV sequences from GenBank and in all the FPV sequences described in this study (detailed below). We conclude, therefore, that the CPV-2 sequence found in H493 presented an intermediate viruslike feature at position 101.

In addition to the six amino acid residues used to differentiate CPV-2 from CPV-2a, three further common mutations that differentiate CPV-2 from CPV-2a strains were found. These mutations were located at amino acid positions 219, 297, and 386. Substitution S297A was first detected in 1987 in CPV-2a strains and is reported to be distributed globally (43). All the CPV-2a isolates from this study had this mutation. Mutations I219V and Q386K were found in all the CPV-2 isolates. Although these two mutations were not found in any of the template strains, they were found in the live-virus vaccine strain contained in the Nobivac Puppy DP vaccine, which has been used in mass dog vaccination programs conducted in the study area (GenBank accession no. MG264079). These replacements (of Ile by Val at position 219 and Gln by lysine at position 386) were patented by the manufacturer (U.S. patent 9,186,398 B2) (44) and introduced in order to attenuate the virus.

Sequence comparisons of the CPV-2 strains obtained in this study showed nucleotide identities of 98.8 to 100% and amino acid identities of 98 to 100%. Two CPV-2

TABLE 4 The 459-amino-acid fragment of the VP2 protein that characterizes carnivore parvoviruses[a]

			Amino acid residue at position:																		
Clade	Strain	Species	20	55	67	81	80	82	99	112	125	134	136	144	156	232	239	241	250	323	401	425
CPV-2	H494	*Canis lupus familiaris*	A	E	R	R	R	V	D	V	D	S	L	E	S	I	D	V	V	N	L	T
	H489															M						
	H504															I		S				
	H506																					
	H469																					
	H499		T											Q								
	H488		T												F		E					P
	H327		T						H													
	H493				T	K		M					F									
	H503																					
	H476*																					
CPV-2a	H491			Q						I		N	M	Q					G			
	H321		T																			
	H501											N										
	H492*																					
	H323*																					
	H318*																					
	H398	*Canis mesomelas*	T				T		H		Y									D		

[a]Amino acid variation is shown. Amino acids used to differentiate CPV-2 from CPV-2a are not included.

nucleotide sequences from different villages and years (H503 from Merenga in 2008 and H506 from Kitawasi in 2005) were identical, and a third sequence (H469 from Merenga in 2008) was translated into the same amino acid sequence. Specific mutations differentiating the strains are detailed in Table 4.

A BLAST search identified similar and identical CPV-2 sequences in different continents. A nucleotide sequence described in a dog in Italy in 2005 (GenBank accession no. FJ222824) (45) was found to be identical to the sequence of H503/H506 and to the Nobivac Puppy DP vaccine strain described in Ecuador (GenBank accession no. MG264079) (46). This is the same vaccine that has been used in mass dog vaccination programs in the Serengeti ecosystem. It was not reported whether the isolate from Italy was collected from a vaccinated or unvaccinated dog. Other sequences containing one of the two patented vaccine strain mutations (I219V and Q386K) were obtained from foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) in China in 2009 (L. Zhang and X. J. Yang, unpublished data [GenBank accession no. GU392236 to GU392241]) and from a dog in the United States in 1995 (GenBank accession no. U22186). All sequences showed a nucleotide identity of 99.8% and an amino acid identity of 99.5% with the sequence of H503/H506.

Sequence comparisons of the CPV-2a strains obtained in this study showed nucleotide identities of 98.6 to 99.5% and amino acid identities of 97.8 to 99.5%. When CPV-2a isolate H501 from this study was compared with two similar strains found in GenBank (from a dog in Italy in 2000 [accession no. AF306445] and a dog in Thailand in 2004 [accession no. FJ869128]), maximum nucleotide identities of 99.8% and 99.6% and a maximum amino acid identity of 100% were obtained. Consequently, the H501 isolate from this study was more similar to the isolates found in Thailand and Italy than to the other two CPV-2a isolates found in Tanzania.

(iii) Phylogenetic analysis in dogs. The phylogenetic tree (Fig. 4) suggests that CPV-2a sequences from this study are closely related to global strains, suggesting that CPV-2a sequences from the Serengeti ecosystem do not form a clear geographic cluster and are closely related to sequences isolated in other continents. Therefore, in contrast to the FPV sequences in wildlife, which displayed localized geographic clustering, the CPV sequences isolated in this study seem to share an evolutionary process with global sequences.

The CPV-2 sequences isolated in this study clustered with two sequences from Italy and China and the Nobivac Puppy DP vaccine strain (GenBank accession no. MG264079).

DISCUSSION

We have demonstrated that carnivore parvoviruses are widely distributed among wild and domestic carnivores in the Serengeti ecosystem. While wildlife was infected with FPV, domestic dogs living around the periphery of the SNP were infected with CPV. With the exception of a jackal infected with CPV-2a, there was no evidence of cross-species transmission, suggesting the existence of two separate epidemiological systems. Given that CPV has been shown to be present in "wilderness" areas in other continents and that cross-species parvovirus transmission has been documented between domestic and captive animals and free-living wild carnivores (5, 6, 47), this finding was unexpected.

Viral populations in wildlife. We found parvovirus DNA in 13.2% of the wild carnivores sampled in the Serengeti ecosystem. The samples were collected through a convenient nonrandom method, and most were collected from animals found dead on the primary road traversing the center of the SNP. As such, this is not likely to be a representative sample and does not provide an unbiased prevalence estimate. However, because the percentage of wildlife samples found to be positive did not change significantly across the 10 years studied or across the species tested, this lends weight to the hypothesis that carnivore parvoviruses are endemic in wildlife species in the Serengeti ecosystem, as may be the case for wildlife in other continents (30). The likelihood of positive samples was not related to proximity to human habitation, which would be consistent with independent routes of carnivore parvovirus transmission in wild and domestic carnivores.

Thirteen wild animal samples were found to be positive for carnivore parvovirus DNA, of which 12 were identified as FPV and 1 was identified as CPV-2a (detected in a black-backed jackal). Important among these results was the detection for the first time of FPV infection in wild dogs, jackals, and hyenas. While many species of Carnivora appear to be susceptible to carnivore parvoviruses, the host range of FPV has been reported to be restricted to foxes, felids, and some closely related families such as mustelids (5; O. Calatayud, F. Esperón, R. Velarde, A. Oleaga, L. Llaneza, A. Ribas, N. Negre, A. de la Torre, A. Rodríguez and J. Millán, submitted for publication). As such, these findings are notable.

In addition, these findings are of interest as they raise questions concerning the interpretation of data from previous serological studies, which assumed that infection with CPV was responsible for seropositive results in jackals, hyenas, and African wild dogs (27, 48). Our results suggest that seropositivity in those previous studies might have resulted from FPV infection instead, highlighting the importance of strain characterization in understanding carnivore parvovirus dynamics.

Host susceptibility to carnivore parvovirus infection is largely driven by the ability of viruses to bind to the carnivore transferrin receptor (TfR) used for host cell attachment (49, 50). Indeed, both FPV and CPV can infect felines because they can bind feline TfR. However, a mutation introduced less than 6 million years ago into the TfR gene encoding the N-linked glycosylation site in the apical domain confers resistance to FPV infection in most canine species. This glycan mutation is present in coyotes (*Canis latrans*), wolves (*Canis lupus*), and domestic dogs (49–51) but has been shown to be lacking in red foxes, bat-eared foxes, and black-backed jackals (51). As predicted by the lack of the glycan-introducing mutation, we report for the first time natural FPV infection in a jackal (H418). Furthermore, we report for the first time that hyenas and African wild dogs can also be infected by FPV, suggesting that these species might also lack the glycan-introducing mutation. This is consistent with the evolutionary history of hyenas, wild dogs, and jackals, which all share a relatively distant common ancestor with wolves, coyotes, and dogs (52, 53). It is possible, therefore, that these species diverged before the emergence of the canid glycan-introducing mutation.

Events of cross-species transmission of FPV among wildlife species have been previously documented in the wild (6, 10) and in captivity (54), suggesting that parvoviruses are transmitted between hosts during contact, for example, predation

and/or scavenging of carcasses. The finding in this study of genetically indistinguishable viruses in sympatric species in the Serengeti ecosystem provides further evidence that FPV can be transmitted between species and that these transmission events occur in this ecosystem. Even where sequence mutations were identified, phylogenetic analysis demonstrated a close relationship among all the sequences described. This clustering is due primarily to two specific mutations (F303Y and I101T) that characterize all identified Serengeti ecosystem strains. The clustering of Serengeti FPV sequences independently of other sequences reported in GenBank and the stable number of infections across the 10-year period studied also suggest that FPV has been present in this ecosystem for a long time and may be endemic.

It is noteworthy that of all the wild carnivore species tested, we detected CPV in only one species, a jackal. This is in contrast to reports describing CPV infection in wild carnivores in other parts of the world (6, 55). Although the Serengeti ecosystem is considered a relatively intact wilderness (56), there are populations of humans and domestic animals, including many dogs, living around its periphery, and incursions frequently occur (57). As a result, it seems likely that wild carnivores would be exposed to CPV in the Serengeti. The lack of detection of CPV in wild carnivores in the Serengeti could arise because wild carnivores are resistant to infection (possibly as a result of FPV within the Serengeti ecosystem creating an immunological barrier), have been clearing infection, or have been dying in small and imperceptible epidemic waves. The latter explanation seems possible given that most parvoviruses causing disease in large cats have been described not as FPV but as CPV (58). Indeed, a recent analysis of long-term serological data to investigate the transmission ecology of CPV in the Serengeti ecosystem indicates that infection cycles in lions are coupled with those in dogs, providing some evidence of cross-species transmission (59). However, as CPV and FPV are antigenically similar and difficult to distinguish serologically, it is likely that seropositive lions were infected with FPV, complicating the interpretation of the serological data. While the genetic analyses provide no evidence for cross-species transmission, the different conclusions drawn by the serological and genetic studies are not mutually exclusive. Our study suggests that it is likely that FPV is circulating as an endemic infection in lions; however, it is also possible that transient outbreaks of CPV may also occur as a result of spillover from domestic dogs. The integration of data from multiple sources and from more comprehensive sampling will clearly be needed to allow a more complete understanding of a complex epidemiological picture.

The detection of carnivore parvovirus DNA in a range of tissues supports the hypothesis that, similar to human parvoviruses (33), carnivore parvovirus DNA remains in the body following initial infection, as has been shown in previous studies (5, 6, 47). These results further highlight the potential value of carnivore parvovirus epidemiological research on tissue samples collected from carcasses and should encourage analysis of such samples collected from other ecosystems across Africa and elsewhere.

Viral populations in dogs and vaccine shedding. With just under half of the domestic dog blood samples being positive, the results indicated that CPV has been circulating widely in the villages adjacent to the SNP during 10 years, which is suggestive of endemicity. This finding was unexpected given that all of the sampled dogs appeared healthy. Our results suggest that CPV DNA persists in blood for longer periods than previously thought (60), with no clinical signs.

Surprisingly, 65% of the sequenced viruses from dogs were CPV-2, even though this strain has been replaced in most areas of the world by the newer antigenic types 2a, 2b, and, more recently, 2c (40). The detection of this strain in several different villages over a 4-year period generates confidence in this finding. Several lines of evidence suggest that a modified live vaccine virus was the source of this CPV-2 strain and that, because all the samples used in this study belonged to unvaccinated individuals, transmission from vaccinated to unvaccinated dogs may have occurred. First, all the CPV-2 sequences described contained two genetic markers patented by the vaccine manufacturer and artificially introduced to attenuate the vaccine virus (44). Second,

three amino acid sequences were identical to the vaccine strain. Third, this vaccine has been used in annual mass dog vaccination programs in the region. Consequently, we conclude that these findings represent cases of natural transmission of vaccine-derived CPV-2 (vdCPV) in domestic dogs. This is the first time that this has been demonstrated empirically.

Although this phenomenon has not been demonstrated previously, the potential for this event and the route of transmission has been reported by previous studies. Two studies demonstrated that 23% of dogs immunized against CPV using a modified live-virus vaccine shed virus DNA in their feces during at least 20 days (60, 61). A third experimental study demonstrated that following contact with vaccinated dogs, unvaccinated dogs became seropositive without showing signs of disease (62). Consequently, it is possible that following transmission of virus from vaccinated individuals, naive dogs are becoming infected. It is also possible that these infections might result in protective immunity against CPV.

Although we did not find evidence of cross-species transmission of vdCPV in the Serengeti ecosystem, the host range might not be restricted to domestic dogs. Indeed, the same artificial mutations have been reported in samples obtained from foxes and raccoon dogs from China in 2009 (Zhang and Yang, unpublished [GenBank accession no. GU392236 to GU392241]), suggesting that vdCPV could be transmitted to wild carnivores. However, because we do not know which brand of vaccine was used in China, we are not able to draw conclusions regarding whether vaccine transmission resulted following vaccination with the Nobivac Puppy DP vaccine or if it can be triggered by the use of other modified live-parvovirus vaccines.

A concern with live vaccine viruses is the potential for reversion to virulence (63, 64), although there are no reports in the literature of this happening with vdCPV. Because recombination (65) or novel mutations might lead to a loss of the benign phenotype, surveillance to monitor for such an eventuality would have merit.

The circulation of vaccine-derived strains CPV-2a, CPV-2b, and CPV-2c would be of more concern than the circulation of the CPV-2 strain because it has been shown that field strains 2a, 2b, and 2c are able to cause disease in felids and in other wildlife species (10, 20). For this reason, vaccine shedding from CPV-2a, -2b, and -2c live vaccines could have an impact on wild carnivores, and we recommend further investigation to assess the risks of the use of these vaccines in proximity to wildlife in protected areas.

Intermediate features. Two sequences from this study showed coding mutations at strain-determinant positions. Intermediate features between FPV and CPV have been described only once in a wild carnivore (a red fox from Germany [66]), and intermediate mutations between different CPV strains were previously described in raccoons (*Procyon lotor*) (55), probably as a result of host adaptation.

The first, a vdCPV from domestic dog H493 sampled in 2009, had the amino acid substitution I101T, common to the CPV-2a strain and to the FPV strains detected in the Serengeti ecosystem. Position 101 is variable, and this mutation was previously described in raccoons and domestic cats (*Felis catus*) (55, 67); however, this is the first time that an intermediate CPV/CPV-2a strain has been reported in dogs (6). Although it is not clear whether this substitution arose after a recombination or a mutation event, an evolving modified live virus is of concern.

The second intermediate mutation was described in the black-backed jackal H398 sequence in 2011. Although we classified this strain as CPV-2a, it presented an Asp replacing an Asn residue at amino acid position 323, which is typical of the FPV strain (40). Amino acid position 323 is located on a raised region of the capsid surrounding the 3-fold spike, which contacts the TfR (50). Although this mutation is predicted to reduce replication in canine cells, it is possible that this viral mutation would favor binding to a feline transferrin receptor present in jackals (51).

In summary, this study has demonstrated that while carnivore parvovirus infection occurs in numerous species living in the Serengeti ecosystem, there appears to be separate transmission routes involving wild and domestic carnivores. Furthermore,

TABLE 5 Taxonomic families and species from which the analyzed samples were obtained[a]

Family and species	No. of isolates													
	Tissue type							Age			Location			
	Sp	Bl	Br	LN	In	Li	SG	Adult	Suba	ND	SNP	NPA	ND	Total tested
Canidae														
Wild dog	11	3	3	3						20	1	9	10	20
Bat-eared fox	10		2	1	1	1		9	2	4	14	1		15
BBJ	5	4	2	1	1	1	1	11		4	13		2	15
Aardwolf	1							1			1			1
Felidae														
Lion	13	25		3	1	2		23	4	17	41	1	2	44
Cheetah	3		2		1			4	1	1	6			6
Leopard	1							1			1			1
Serval	1							1			1			1
Hyaenidae														
Spotted hyena	8	8	7	6	2	1		13	9	10	30	1	1	32
Striped hyena			1	1				2			1	1		2
Viverridae														
African civet	5							5			4		1	5
Genet	3							2		1	1	1	1	3
Herpestidae														
Mongoose	2		2		2	1		5	2		5	1	1	7
Total	63	40	19	15	8	6	1	77	18	57	119	15	18	152

[a]Sp, spleen; Bl, blood; LN, lymph node; In, intestine; Li, liver; SG, salivary gland; Suba, subadult; ND, no data; SNP, Serengeti National Park; NPA, neighboring protected areas; BBJ, black-backed jackal.

while FPV appears endemic in wild carnivore populations living in the Serengeti ecosystem (including canids and hyenas), CPV-2 and CPV-2a appear to be circulating almost exclusively in domestic dog populations, with CPV-2 infection likely arising as a result of vaccine shedding.

MATERIALS AND METHODS

Sampling. Archived (−20°C) biological samples collected between 2002 and 2011 were used in this study. These were as follows: (i) tissue samples (n = 112) collected during wild carnivore postmortem examinations carried out in the Serengeti National Park (SNP) (linked GPS coordinate data were available for most of the samples, and the cause of death for each of the sampled wild carnivores was unknown); (ii) blood samples (n = 40) collected opportunistically from wild carnivores during general anesthesia immobilization; and (iii) uncoagulated (EDTA) blood samples (n = 62) collected during mass dog vaccination programs from manually restrained, healthy, unvaccinated (<1.5 years old) domestic dogs living in villages around the periphery of the SNP (these villages were Nyamburi, Merenga, Pinyinyi, Malambo, Kitawasi, Engarasero, Arash, and Piyaya) (Fig. 5).

The taxonomic family, tissue type, age, and geographic provenance of each sample are detailed in Table 5.

One lion (*Panthera leo*) fecal sample was available for testing. It belonged to a spleen-positive adult animal (H440) and was excluded from the statistical analysis.

TABLE 6 Primers used in this study

Primer	Sequence	Binding site (nt)[a]
Forward (68)	5′-TGGAACTAGTGGCACACCAA-3′	3456–3473
Reverse (68)	5′-AAATGGTGGTAAGCCCAATG-3′	3636–3655c
Probe (68)	5′-CAGGTGATGAATTTGCTACAGG-3′	3555–3576
VPF (75)	5′-ATGGCACCTCCGGCAAAGA-3′	2285–2303
VPR (75)	5′-TTTCTAGGTGCTAGTTGAG-3′	4512–4530c
P1 (76)	5′-ATGAGTGATGGAGCAGTTC-3′	2788–2807
P3 (76)	5′-CCATTTCTAAATTCTTTG-3′	3752–3770
P4 (76)	5′-AAGTCAGTATCAAATTCTT-3′	4202–4221c

[a]Nucleotides (nt) numbered according to Reed et al. (69). c, complementary.

TABLE 7 PCR thermal cycling conditions used in this study

Reaction	Primers	Cycling conditions	Amplicon length (nt)
qPCR	Forward(68), reverse(68), probe(68)	5 min at 95°C and 40 cycles (30 s at 95°C and 30 s at 60°C)	163
External PCR	VPF(78), VPR(78)	5 min at 94°C, 40 cycles (30 s at 94°C, 30 s at 48°C, and 150 s 72°C), and 7 min at 72°C	2,209
Internal PCR I	P1(79), reverse	5 min at 94°C, 40 cycles (15 s at 94°C, 15 s at 58°C, and 110 s at 72°C), and 7 min at 72°C	829
Internal PCR II	P3(79), P4(79)	5 min at 95°C, 30 cycles (30 s at 95°C, 30 s at 44°C, and 45 s at 72°C), and 7 min at 72°C	432
Internal PCR IV	Forward, P4	5 min at 94°C, 40 cycles (30 s at 95°C, 30 s at 50°C, and 96 s at 72°C), and 7 min at 72°C	746

Molecular analysis. DNA was purified from 50 mg of tissue or 200 μl of blood in a series of rapid "wash-and-spin" steps, using the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. Purified DNA was stored at −80°C until further use.

To determine the presence of parvovirus DNA (case ascertainment), a previously described real-time PCR method (68) (quantitative PCR [qPCR]) was performed. It targeted a conservative parvovirus region of 163 nucleotides, and it did not discriminate between different parvovirus strains. The primers used in this study are detailed in Table 6, and the thermal cycling conditions are detailed in Table 7. In this qPCR, the final mixture of 25 μl contained 0.4 μM primers, 10 μl of Quantiprobe (Qiagen GmbH, New York, NY, USA), 0.4 μM probe, 3.5 μl of template DNA, and nuclease-free water. The assay was performed on a StepOne real-time PCR system.

For the characterization of the carnivore parvovirus strains, a 1,377-nucleotide portion of the *vp2* gene (total length, 1,755 bp) was amplified with four nested PCRs. The final mixture of 25 μl contained 0.5 μl of primers, 0.25 μl of FastStart universal master mix (Roche), 1.5 μl of Cl_2Mg, 2.5 μl of 10× buffer, 0.5 μl 10 μM deoxynucleoside triphosphate (dNTP), 4 μl of template DNA, and sterile nuclease-free water. Three internal nested PCRs amplified three segments of different lengths (Table 6), which were subsequently purified. Five primers (P1, P3, P4, forward, and reverse [Table 6]) were used to sequence five overlapping fragments covering a total of 1,377 nucleotide residues with the automated Sanger sequencing method.

A 10^{-2} commercial CPV vaccine dilution (Nobivac Puppy DP; MSD Animal Health, Carbajosa de la Sagrada, Spain), containing attenuated live CPV-2, was added as a positive control, and sterile nuclease-free water was added as a negative (blank) control for each step of the molecular analysis.

Sequence data. BLAST searches in GenBank were performed for each sequence generated. Nucleotide sequences were translated into putative amino acid sequences, and position sites were numbered (69). Specific amino acids were used for classification of the FPV, CPV-2, CPV-2a, CPV-2b, and CPV-2c strains (20, 38, 39). Nucleotide and amino acid sequence pairwise identity was calculated using the online software SIAS (Sequence Identity and Similarity) (70). Only sequences of >1,300 bp were used in this analysis. In addition, template sequences of carnivore parvoviruses were randomly selected from 1990, 2007, and 2015 and were included for comparison (GenBank accession no. M38246, EU145593, and KX434462). The 1990 sequence was the oldest one found in the GenBank database and was included in the study to assess viral diversity over time.

Sequences were aligned with the ClustalW method using MEGA7 software (71). A maximum likelihood phylogenetic tree was inferred and reliability was evaluated with the bootstrap method based on 1,000 replicates using the same software.

Statistical analysis. Statistical analyses were performed using the exact binomial confidence interval (95% confidence level) for prevalence calculations. Associations between the presence of carnivore parvovirus DNA and potential explanatory variables, such as type of tissue, species, family, age (young, juvenile, or adult), sex, and year of collection, were evaluated by binomial logistic regression using R software (72). Variable selection was carried out using manual forward selection based on the lowest Akaike information criterion (AIC) value. Strengths of associations were determined based on odds ratios with 95% confidence limits (73). Wildlife sample coordinates were used to calculate the distance (kilometers) from the location of sampling of wildlife to the point of nearest human contact, with the locations of (i) the nearest building and (ii) the SNP boundary used as proxy measures. QGIS Geographic Information System software (74) was used for distance calculations and for the representation of sample locations. A binomial logistic regression model was constructed with the proxy measures described above as predictor variables to investigate whether proximity to human habitation and/or the park boundary predicted the likelihood of carnivore parvovirus infection.

Data availability. Sequences described in this study were submitted to GenBank (accession no. MK251434 to MK251461).

ACKNOWLEDGMENTS

We acknowledge the Tanzania Ministry of Livestock and Fisheries, Tanzania National Parks (TANAPA), the Tanzania Wildlife Research Institute (TAWIRI), the Ngorongoro Conservation Area Authority, and the Tanzania Commission for Science and Technology for permissions and the TANAPA Veterinary Unit, the TAWIRI-Messerli Foundation Wildlife Veterinary Programme, the Serengeti Lion Project, the

Frankfurt Zoological Society, and district veterinary offices in Serengeti and Ngorongoro Districts for assistance with field activities. We thank the Animal Health Research Center (CISA), the National Institute for Agriculture and Food Research and Technology (Valdeolmos, Madrid, Spain), and the Nelson Mandela African Institution of Science and Technology (Arusha, Tanzania) for their support with the laboratory analysis. We are also grateful to Léon Abraham Sancha and Kennedy Elisha Jomanga for their support.

The sample collection component of this work was initially supported by the joint National Institutes of Health (NIH)/National Science Foundation (NSF) Ecology of Infectious Diseases Program under grant no. NSF/DEB0225453, and we acknowledge later significant support provided by Lincoln Park Zoo, Washington State University, the Tusk Trust, the World Society for the Protection of Animals, Paradise Wildlife Park, and MSD Animal Health (formerly Intervet and Intervet Schering-Plough). We are extremely grateful to the Morris Animal Foundation, which provided the funding for the sample processing, laboratory and data analysis, and manuscript writing components of this project (grant no. D16ZO-819). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. We declare that there is no conflict of interest.

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